Collaborative Study of the Extraction of Plant Sterols from Adulterated Butter Oil Using a Digitonin-Impregnated Celite Column

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A rapid screening method for the analysis of the phytosterol, β-sitosterol, in butter oil adulterated with vegetable oil has been studied collaboratively. The sterols are removed from the adulterated butter oil by passing the sample through a digitonin-impregnated Celite 545 column, eluting the sterols with dimethyl sulfoxide, and analyzing the eluate for β-sitosterol by gas-liquid chromatography using a 3% JXR column. The average coefficient of variation for those samples containing more than 4 mg β-sitosterol/100 g adulterated butter oil is 12.6%. Therefore, β-sitosterol can be used as an index to qualitatively detect vegetable oil adulteration of butter oil.

At the present time there is no rapid AOAC method for the determination of vegetable oil adulteration of butter oil. The method described in this paper is designed as a rapid screening method for analysis of β-sitosterol in adulterated butter oil. The method was first reported by Katz and Keeney (1), based on the digitonin-impregnated column procedure of Schwartz *et al.* (2). LaCroix (3) demonstrated the suitability of this procedure and a collaborative study was recommended based on the results of the investigation.

Collaborative Study

Each of 8 collaborators was sent 10 samples of butter oil obtained from the USDA Dairy Products Laboratory, Beltsville, Md. 20705. Nine of the samples contained 4 vegetable oils at 2 levels of adulteration, and 1 sample contained no vegetable oil. One of the samples was designated as a practice sample of stated β-sitosterol composition. The collaborators were instructed to obtain

satisfactory results on the known sample, using the test method, before proceeding with the unknown sample. Some precautionary remarks were included in the instructions. Collaborators were also instructed to report any difficulties and commentary associated with the method.

METHOD[1]

(Applicable to samples contg ≥4 mg free β-sitosterol/100 mg butter oil)

28.075 *Principle*

Free 3-β-OH sterols are removed from butter oil by complexing with digitonin and sterols are then removed from digitonide-Celite column by elution with dimethyl sulfoxide (DMSO). Butter oil has range of 0–1 mg β-sitosterol/100 g and ice cream has apparent value of ca 4 mg/100 g fat from emulsifiers.

28.076 *Reagents*

(a) *Diatomaceous earth.*—Celite 545, or equiv.

(b) *Digitonin.*—(Mann Research Laboratories, Mountain View Ave, Orangeburg, NY 10963).

(c) *β-Sitosterol std soln.*—2 μg β-sitosterol/μl $CHCl_3$. Prep. from Aldrich Chemical Co., 2371 N. 30th St, Milwaukee, WI 53210, reagent (64% β-sitosterol, 36% campesterol) or Applied Science Laboratories, Inc. reagent (90% β-sitosterol, 10% campesterol).

(d) *n-Hexane.*—Distill pure grade over KOH. (*Caution: See* **46.011, 46.037, 46.039,** and **46.061.**)

28.077 *Apparatus*

(a) *Gas chromatograph.*—Operating conditions: temps, column 225–245° and injection port and flame ionization detector 265–285°. Adjust N carrier gas flow (ca 50–60 ml/min) to obtain following retention times: cholesterol 16–18 min, campesterol 22–24 min, and β-sitosterol 28–30 min. Use 6′ × 4 mm id column contg 3% JXR silicone on 100–120 mesh Gas Chrom Q, or equiv., prepd as in **28.073(b)**, and condition column 24 hr at 250° with 15–20 psi N.

(b) *Performance.*—Monitor performance of gas chromatograph by noting sepn of campesterol and sitosterol expressed as peak resolution = $2D/(C + B)$, where D = distance between the 2 peak maxima, C = campesterol peak base width, and B = β-sitosterol peak base width. Peak resolution should be ≥1.6.

(c) *Injection technic.*—With 10 μl Hamilton microsyringe, draw 1 μl air into barrel, insert needle into soln, and draw desired amt into barrel. Remove needle from soln and draw 1 μl air into barrel. Note vol. on scale and adjust to desired vol., if necessary.

(d) *Preparation of std curve.*—Prep. std soln of 2 μg β-sitosterol/μl $CHCl_3$. (Det. composition of std as in **28.072(e)**.) Obtain std curves daily covering range 1–10 μg β-sitosterol, using ≥3 points. Plot area of β-sitosterol peak against μg β-sitosterol.

28.078 *Preparation of Column*

Dissolve, with heating, 300 mg digitonin in 5 ml H_2O, add to mortar and pestle contg 10 g Celite, and mix thoroly. (Packing material can be kept several months if stored at 5° in tightly closed container.) Transfer 3 g Celite-digitonin mixt. to 2 × 12 cm column and pack firmly, using packing rod. (Flow rate of tightly packed column is 0.5–0.75 ml/min.) Sat. column with 5 ml *n*-hexane and let flow thru packing until *n*-hexane reaches top of packing material. Use column immediately. Do *not* let dry.

28.079 *Preparation of Sample*

Dissolve 900 mg butter oil in 3 ml *n*-hexane. Quant. transfer soln, using disposable pipet, to digitonin-Celite column and let pass thru column until soln has entered packing material. Wash sample beaker twice with 2 ml *n*-hexane and add each wash to column, rinsing column sides. Wash column with five 2 ml portions *n*-hexane. After all hexane has entered column, wash with five 2 ml portions benzene. After last portion benzene reaches top of packing material, stop flow and wash column tip thoroly with benzene to remove traces of fat. (Failure to wash column sides and column tip with solv. will result in poor chromatograms due to interference from triglycerides.) Discard hexane and benzene. Elute sterols with 10 ml DMSO and collect entire eluate in 15 ml screw-cap centrf. tube.

Add 3 ml *n*-hexane to eluate, shake, and centrf. Transfer upper layer contg sterols to second screw-cap centrf. tube. Repeat extn of DMSO layer in first tube with two 4 ml portions *n*-hexane-benzene (1 + 1), carefully transferring upper layer to second tube each time. Vigorously shake pooled upper layers with 3 ml H_2O and centrf. until clear. Remove upper layer and evap. under N or filtered air in 30 ml beaker on steam bath. Transfer residue to 0.5 dram screw-cap vial with two 0.8 ml portions $CHCl_3$. After evapg solv. with N or filtered air over steam bath, redissolve sterols in 0.1 ml $CHCl_3$ for GLC analysis.

28.080 *Determination*

Inject 2–8 μl extd sample and calc. β-sitosterol by converting peak area to wt, using daily std curve.

[1] The secton numbers within the method are those for the 11th ed. of *Official Methods of Analysis*, 1970 secs. **28.072**(e) and **28.073**(b), see p. 627 (4). Cautionary notes, if present, refer to the new chapter on safety, Chapter 46.

Table 1. Collaborative results for β-sitosterol content of adulterated butter oil (mg β-sitosterol/100 g butter oil)

Lab.	5% Adulteration by: 1. Cottonseed Oil	2. Soybean Oil	3. Safflower Oil	4. Peanut Oil	2% Adulteration by: 5. Cottonseed Oil	6. Soybean Oil	7. Safflower Oil	8. Peanut Oil	9. Butter Oil Blank	10. Known Peanut Oil (5%)
A	13.9	6.7	3.1	8.2	5.9	3.3	2.6	4.4	trace	8.3
B	13.9	6.4	4.2	8.5	6.2	1.9	1.1	2.3	trace	8.2
C[a]	11.3	7.0	3.2	8.2	4.6	2.9	1.1	3.0	0	8.1
D[a]	13.9	6.1	5.1	7.9	7.0	4.2	3.2	4.0	0	7.4
E[a]	13.9	5.6	4.9	9.2	6.9	2.8	3.3	4.3	0	7.2
F[a]	15.9	5.7	3.9	6.1	5.7	2.5	1.9	2.6	trace	7.1
G[a,b]	20.0	8.7	8.3	10.9	8.8	3.7	3.5	4.6	trace	9.7
Mean	13.8±1.4	6.3±0.54	4.1±0.8	8.0±1.0	6.1±1.0	2.9±0.8	2.2±1.0	3.4±0.9	0	7.8±0.7
Coeff. of var.	10.2%	8.6%	19.6%	12.5%	16.2%	36.2%	45.4%	37.8%	0	8.8%

[a] Average of two determinations.
[b] Eliminated by Youden's ranking test.

Calc. mg β-sitosterol/100 g butter oil = (μg from curve/1000) × (100/μl injected) × (100/g sample).

Identify peaks from butter oil samples by comparing their retention time to retention time of known compds. Relative retention times are cholesterol 1.0, campesterol 1.4, and β-sitosterol 1.7.

Results and Discussion

The results obtained by the collaborators are summarized in Table 1. Those results which were reported in duplicate for each sample were averaged and 1 value was reported in the table. Seven of 8 laboratories submitted reports. However, Laboratory G reported consistently high values on all samples; those results were omitted on the basis of the ranking test (5).

All the collaborators reported difficulty with quantitation of samples containing less than 4 mg β-sitosterol/100 mg butter oil, although the β-sitosterol could be qualitatively observed. Since the level of β-sitosterol present in samples 6, 7, and 8 was near the operational sensitivity limits of the instruments used by the collaborators, the high coefficient of variation of these samples is not surprising in view of these comments. The overall coefficient of variation of those samples containing 4 mg or more β-sitosterol/100 mg butter oil is 12.6%.

All values in Table 1 which were not rejected by the ranking test were subjected to the Dixon outlier test (6). No values were eliminated.

Also, the β-sitosterol content of butter oil adulterated with safflower oil proved to be the most difficult to quantitate due to the low β-sitosterol content as well as the presence of other sterol peaks which are in close proximity to β-sitosterol (1, 7, 8).

Figure 1 shows the GLC pattern of the sterols of cottonseed oil and butter oil adulterated at

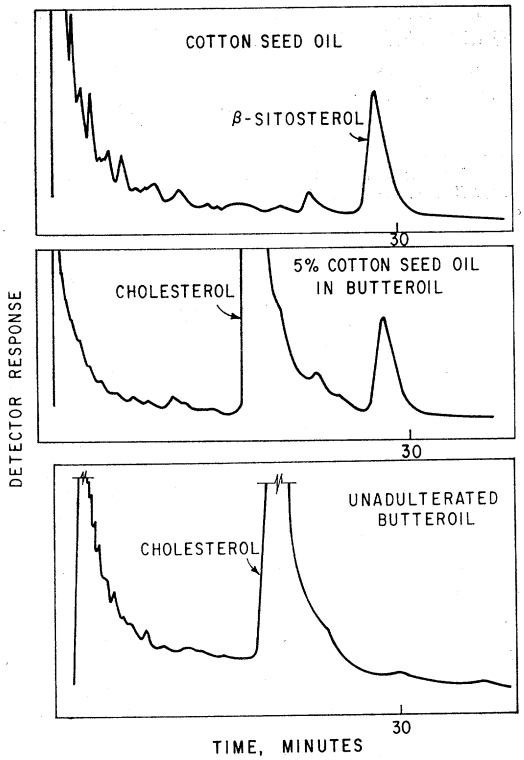

FIG. 1—GLC patterns of sterols of cottonseed oil, butter oil adulterated with cottonseed oil, and unadulterated butter oil.

5% with cottonseed oil and the GLC pattern of unadulterated butter oil. GLC analysis of 4 market brands of butter oil, as well as several preparations of butter oil prepared at the USDA Dairy Products Laboratory, showed that little or no β-sitosterol could be observed. Therefore, the presence of a sizable β-sitosterol peak would provide a suitable index of vegetable oil adulteration of butter oil.

The identification of some vegetable oils by their sterols has been published (1, 7, 8). Thus, in addition to detecting low levels of adulteration, correlation of the total sterol GLC pattern could be used as a means of identifying some types of oils used as adulterating agents.

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REFERENCES

(1) Katz, I., and Keeney, M., *J. Dairy Sci.* **50**, 1754–1768 (1967).
(2) Schwartz, D. P., Brewington, C. R., and Burgwald, L. H., *J. Lipid Res.* **8**, 54–55 (1967).
(3) LaCroix, D., *JAOAC* **52**, 600–602 (1969).
(4) Thorpe, C. W., Pohland, L., and Firestone, D., *JAOAC* **52**, 774–778 (1969).
(5) Youden, W. J., *Statistical Techniques for Collaborative Tests*, AOAC, Washington, D.C., 1967.
(6) Dixon, W. J., and Massey, F. J., *Introduction to Statistical Analysis*, 2nd Ed., McGraw-Hill, Inc., New York, 1967.
(7) Eisner, J., and Firestone, D., *JAOAC* **46**, 542–550 (1963).
(8) Eisner, J., Iverson, J. L., and Firestone, D., *JAOAC* **49**, 580–590 (1966).

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